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Genetic ablation of β-catenin inhibits the proliferative phenotype of mouse liver adenomas

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Background: Aberrant activation of Wnt/β-catenin has been implicated in various cancer-related processes, for example, proliferation or tumour cell survival. However, the exact mechanism by which β-catenin provides liver tumour cells with a selective advantage is still unclear. This study was aimed to analyse growth behaviour and survival of β-catenin-driven mouse liver tumours after β-catenin ablation.

Methods: Transgenic mice with a controllable hepatocyte-specific knockout of *Ctnnb1* (encoding β-catenin) were generated and liver tumours were induced by means of a *N*-nitrosodiethylamine/phenobarbital tumour initiation/promotion protocol, which leads to the outgrowth of hepatocellular tumours with activated β-catenin. Cre recombinase was activated and the effects of the knockout in the tumours were studied.

Results: Activation of Cre recombinase led to the knockout of *Ctnnb1* in a fraction of tumour cells, thus resulting in the formation of two different tumour cell subpopulations, with or without β-catenin. Comparative analysis of the two subpopulations revealed that cell proliferation was significantly decreased in *Ctnnb1*-deleted hepatoma cells, compared with the corresponding non-deleted cell population, whereas no increased rate of apoptosis after knockout of *Ctnnb1* was observed.

Conclusions: β-catenin-dependent signalling is an important regulator of hepatoma cell growth in mice, but not a crucial factor in the regulation of tumour survival.

Up to 30% of human hepatocellular carcinomas and 80% of hepatoblastomas display aberrant activation of the Wnt/β-catenin signalling pathway (de La Coste *et al*, 1998; Lopez-Terrada *et al*, 2009; Schmidt *et al*, 2011). This is mainly caused by genetic alterations in the *CTNNB1* gene, encoding β-catenin, an important transcription factor in canonical Wnt signalling. Mutations in *CTNNB1* lead to amino acid exchanges near the N terminus of the β-catenin protein where important phosphorylation sites are located, which are essential for its proteasomal degradation. On accumulation in the cytoplasm, mutant β-catenin translocates into the nucleus to associate with T-cell factor/lymphoid enhancer factor proteins and to promote the expression of target genes (Lustig and Behrens, 2003).

In mouse liver, tumours harbouring a mutationally activated Wnt/β-catenin signalling pathway can be induced by an initiation/promotion regimen according to Moennikes *et al* (2000): 6-week-old mice are given a single dose of the liver carcinogen *N*-nitrosodiethylamine (DEN) followed by chronic treatment with phenobarbital (PB). Using this regimen, the tumour promoter PB selects for the outgrowth of *Ctnnb1*-mutated hepatoma cells (*Ctnnb1* is the mouse orthologue to *CTNNB1* in humans) and the frequency of liver tumours mutated in *Ctnnb1* is about 80% (Aydinlik *et al*, 2001).

There is evidence that aberrant β-catenin-dependent signalling promotes cell proliferation and inhibits apoptosis, thus providing cancer cells with a selective advantage. Expression of an oncogenic

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form of β-catenin leads to hepatomegaly in transgenic mice due to increased hepatocyte proliferation (Cadoret *et al*, 2001). Furthermore, several known target genes of β-catenin are implicated in cell cycle progression, such as cyclin D1 and c-myc (He *et al*, 1998; Shtutman *et al*, 1999), or in the regulation of apoptosis (Zhang *et al*, 2001). Activation of β-catenin in different cell lines results in increased proliferation, while at the same time inhibition of apoptosis is observed (Orford *et al*, 1999; Shang *et al*, 2004). Aberrant Wnt signalling is sufficient to generate intestinal lesions and adenomas (Shibata *et al*, 1997; Romagnolo *et al*, 1999) and is also required for maintenance of colorectal tumour xenografts in mice (Scholer-Dahirel *et al*, 2011). In liver, β-catenin-positive cell islets, which have evaded the hepatocyte-specific knockout of *Ctnnb1* in respective transgenic mice, show a higher proliferative index in presence of PB and in livers with a pre-cirrhotic phenotype, as compared with surrounding hepatocytes lacking β-catenin (Braeuning *et al*, 2010). This suggests that, at least under certain conditions, β-catenin provides a growth advantage to hepatocytes, thereby contributing to liver carcinogenesis. Respective cell islets also exhibit reduced levels of the gap junction-forming protein connexin 32 (Cx32), similar to what had been observed earlier in *Ctnnb1*-mutated liver tumours (Moennikes *et al*, 2000; Marx-Stoelting *et al*, 2008) Thus, inhibition of cell–cell communication might be involved in the regulation of hepatoma cell proliferation by β-catenin.

In a study conducted by Malanchi *et al* (2008), transgenic mice (referred to as K14-creER^{T2}:β-catenin$^{lox/lox}$ mice) were used to investigate the role of β-catenin in established chemically induced skin tumours with increased Wnt/β-catenin signalling. Expression of Cre recombinase under the control of the *keratin 14* (K14) locus resulted in the deletion of *Ctnnb1* in epidermal cells and subsequent complete tumour regression within 5–6 weeks, clearly indicating the important role of the signalling protein for tumour cell survival in this model.

Despite the impressive results from the above-mentioned experiment, the relevance of β-catenin activation for proliferation and survival of tumour cells in liver is not well understood. We have therefore now performed an experiment similar to that of Malanchi *et al* (2008), using a genetically modified mouse line that enabled us to study the effect of a tamoxifen-induced, hepatocyte-specific knockout of *Ctnnb1* during chemical hepatocarcinogenesis. The results of our study show that *Ctnnb1* ablation negatively affects liver tumour cell proliferation but has no significant influence on their survival.

MATERIALS AND METHODS

Animal breeding. Transgenic *Ctnnb1*$^{loxP/loxP}$ mice carrying loxP sites flanking the exons 3 and 6 in the *Ctnnb1* gene (Huelsken *et al*, 2001) were interbred with TTR-Cre-Tam mice expressing a tamoxifen-inducible modified Cre recombinase (MerCreMer) under the control of the hepatocyte-specific transthyretin promoter (Tannour-Louet *et al*, 2002; Anson *et al*, 2012) to obtain *Ctnnb1*$^{loxP/loxP}$, TTR-Cre-Tam mice (Figure 1A). Genotyping of mice for *Ctnnb1*$^{loxP/loxP}$ and *Cre* was performed by standard PCR as recently described (Braeuning *et al*, 2009). Mice carrying *Ctnnb1*$^{loxP/loxP}$ and one *Cre* allele are referred to as '*Ctnnb1* KO mice' in the following text, the respective *Cre*-null mice are called '*Ctnnb1* WT mice', as they are phenotypically normal. Mice were kept on a 12-h dark/light cycle and had access to food and tap water *ad libitum*. Animals received humane care and protocols complied with institutional guidelines.

Animal experiment. For the induction of liver tumours (Figure 1B), we followed a standard initiation/promotion protocol (Moennikes *et al*, 2000). Six-week-old male C3H *Ctnnb1* WT ($n=16$) and KO ($n=35$) mice were given a single intraperitoneal injection of DEN (90 μg g^{-1} body weight; dissolved at 9 mg ml^{-1} in 0.9% NaCl solution, injected volume 10 μl g^{-1} body weight). After a treatment-free interval of 3 weeks, mice were kept on a diet containing 0.05% PB (Ssniff, Soest, Germany) for a time period of 25 weeks. After 1 week on a PB-free control diet, mice were then given five intraperitoneal injections of each 1.5 mg tamoxifen for 5 consecutive days. Tamoxifen (Sigma, Taufkirchen, Germany) was dissolved in ethanol (67 mg ml^{-1}) and further diluted in corn oil to a final concentration of 10 mg ml^{-1} as previously described (Ganzenberg *et al*, 2013). Mice were killed 1–7 weeks after the last tamoxifen treatment. 5-bromodeoxyuridine (BrdU; AppliChem, Darmstadt, Germany) was dissolved in the drinking water (1 mg ml^{-1}) and given orally to the animals for 72 h before killing. Killing was always between 0900 and 1100 h to avoid circadian influences. Livers were excised and the number and size of macroscopically visible tumours were recorded. Liver aliquots were frozen on dry ice and stored at −70 °C for further analyses or were fixed in 4% paraformaldehyde and embedded in paraffin for the preparation of TUNEL (TdT-mediated dUTP nick end labelling) stainings (see section below).

Immunohistochemical staining. Slices from frozen livers (10 μm thickness) were prepared and immunohistochemically stained as previously described (Braeuning *et al*, 2010), using antibodies against glutamine synthetase (GS; 1 : 1000, Sigma), β-catenin (1 : 50, Cell Signaling, Danvers, MA, USA) or BrdU (1 : 50, Dako, Glostrup, Denmark) in combination with horseradish peroxidase-conjugated secondary antibodies directed against rabbit (1 : 100, Dako) or mouse (1 : 20, Sigma) immunoglobulins with 3-amino-9 ethylcarbazole/H_2O_2 as substrates. For double staining of GS and Cx32, GS was visualised by the use of β-galactosidase-conjugated secondary antibodies (1 : 50, American Qualex, San Clemente, CA, USA) in combination with an antibody against Cx32 (1 : 250, Invitrogen/Zymed, Darmstadt, Germany) and horseradish peroxidase-conjugated secondary antibodies directed against rabbit (1 : 100, Dako) immunoglobulins with 3-amino-9-ethylcarbazole/H_2O_2 as substrates.

Tumour analyses. An Axio Imager light microscope (Imager.M1, Zeiss, Göttingen, Germany) with Axiovision Rel. 4.5 software (Zeiss) was used for acquisition of photographs and for further analysis of tumours. Counting of GS- and BrdU-positive cells within tumour subpopulations, grading of Cx32 levels and determination of tumour area fractions, which are equivalent to the tumour volume fractions, were performed using images of stained liver sections.

PCR analyses. Liver slices were stained for GS and respective tumour areas were punched out using a sharpened cannula. The tissue samples were digested with proteinase K and DNA was amplified by standard PCR methods. Tamoxifen treatment-induced Cre-mediated recombination was verified by screening for deletion of the loxP-flanked parts within the *Ctnnb1* gene of four representative tumours (Figure 1C; for further details see Huelsken *et al* (2001)). Hot spot mutations in exon 3 of the *Ctnnb1* gene in GS-positive tumours were detected by standard sequencing (Braeuning *et al*, 2010).

TUNEL assay. Apoptotic cell death was determined by TUNEL staining using the *In Situ* Cell Death Detection Kit, POD (Roche, Mannheim, Germany) according to the manufacturer's instructions for paraffin-embedded tissue sections. To induce DNA strand breaks in positive controls, sections were incubated with benzonase nuclease (Sigma) before labelling procedures.

Statistical analyses. The percentages of BrdU-labelled tumour cells were determined for the GS-negative and -positive tumour cell subpopulations within each tumour and the paired Student's *T*-test

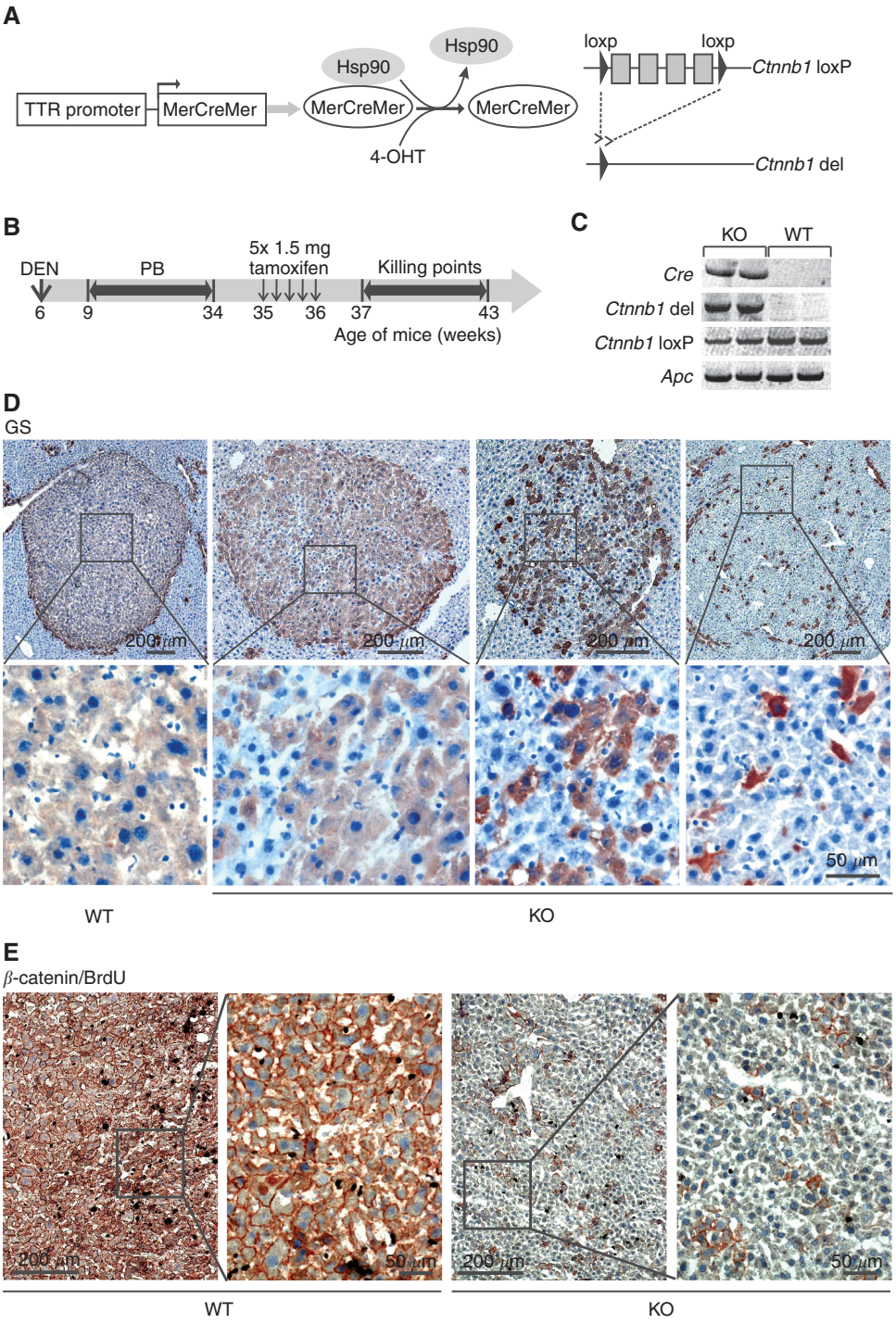

Figure 1. Deletion of β-catenin in β-catenin-activated mouse liver tumours. (**A**) Schematic representation of the *Ctnnb1* gene in livers of transgenic mice. Expression of the modified Cre protein MerCreMer is under the control of the liver-specific transthyretin (TTR) promoter. In the presence of the tamoxifen metabolite 4-hydroxytamoxifen (4-OHT), Cre recombinase, which is flanked by modified ligand-binding domains of the mouse oestrogen receptor (Mer), is released from its chaperones (heat shock protein 90, Hsp90) and targets loxP sites flanking the exons 3 and 6 within the *Ctnnb1* gene. Cre-mediated recombination results in a deleted allele encoding a non-functional β-catenin protein. (**B**) Schematic delineation of experimental setup: mice received a single intraperitoneal (i.p.) injection of *N*-DEN (90 $\mu g\, g^{-1}$ body weight) at 6 weeks of age. Subsequently, mice were continuously fed a diet containing 0.05% PB to select for *Ctnnb1*-mutated hepatomas. After a treatment-free period of 1 week, deletion of the *Ctnnb1* gene in KO mice was achieved by i.p. injection of 1.5 mg tamoxifen for 5 consecutive days. Animals were then killed at different time points after application. (**C**) PCR analysis of tumour DNA from two representative *Ctnnb1* KO and WT mice. The recombined, deleted *Ctnnb1* gene was detected exclusively in Cre-expressing KO mice after tamoxifen treatment. Non-recombined $Ctnnb1^{loxP/loxP}$ was detected in both *Ctnnb1* KO and WT mice but with lower amounts in Ctnnb1 KO mice. The *Apc* gene was amplified as a reference gene. (**D**) Immunostaining for GS, a marker for β-catenin activation. *Ctnnb1*-mutated tumours from WT animals show uniform expression of GS (left images). After KO of *Ctnnb1*, GS-negative hepatoma cells are observed within the tumours. The number of residual GS-positive cells varies between different tumours. (**E**) Tumours from *Ctnnb1* WT and KO mice double stained for β-catenin and BrdU. β-catenin is present at the cell membrane of all tumour cells from WT mice, whereas only single cells possess β-catenin in tumour tissue from a respective KO animal.

was used for statistical analyses. Differences were considered significant when $P<0.05$. Statistical significance is indicated by asterisks (*$P<0.05$; ***$P<0.001$).

RESULTS

Tamoxifen-induced recombination of *Ctnnb1* in transgenic mice. Following the induction of Cre recombinase by tamoxifen according to Ganzenberg *et al* (2013), PCR analyses of tumour tissue samples demonstrated *Ctnnb1* deletion exclusively in the Cre-positive mice. Accordingly, the levels of non-recombined floxed *Ctnnb1* were reduced in these mice (Figure 1C). Residual floxed *Ctnnb1* in tumour cells from KO animals may derive from the non-parenchymal cells not expressing Cre, or from incomplete recombination in the hepatoma cells. Hepatic tumour burden (measured as the tumour volume fraction) at the time point of tamoxifen injection was ~3%, as can be estimated from the observed tumour volume determined 1 week later at the time of killing of the first study group (compare Figure 2B).

Tumour phenotype after tamoxifen-induced KO of *Ctnnb1*. Morphologically, the majority (~90% in number and size) of liver tumours were eosinophilic, well-differentiated hepatocellular adenoma. Expression of the direct β-catenin target GS is one of the most reliable markers for β-catenin activation in murine liver. It is homogeneously expressed in chemically induced mouse liver tumours and there is an almost 100% concordance between activated β-catenin and GS expression (Loeppen *et al*, 2002; Chafey *et al*, 2009). Furthermore, GS is absent from *Ctnnb1* KO hepatocytes (Braeuning *et al*, 2010). To confirm the correlation between high GS expression and activating mutations in the *Ctnnb1* gene, tumour mutation analyses were performed. Twelve out of 13 analysed GS-positive tumours (92.3%) from *Ctnnb1* WT (5 out of 5 tumours *Ctnnb1* mutated) or KO (7 out of 8) animals were point mutated in the hot spot regions of the *Ctnnb1* gene.

Immunostains of tumours from tamoxifen-treated *Ctnnb1* WT mice revealed homogeneous GS expression throughout the tumours (Figure 1D, left image), indicative of active β-catenin-dependent signalling. Accordingly, high levels of the β-catenin protein were present in the respective GS-positive tumour cells (Figure 1E). In contrast, tumours from KO animals were composed of a mixture of GS-negative and GS-positive tumour cell subpopulations (Figure 1D, images 2–4). Of note, immunostaining for β-catenin was mostly membranous, as can be expected from chemically induced mouse liver tumours that contain rather low levels of nuclear β-catenin (Devereux *et al*, 1999; Aydinlik *et al*, 2001; Braeuning *et al*, 2007). The proportion of GS-negative and -positive cells was at great variance between individual tumours, and no clear-cut correlation between the time point of killing and the proportion of GS-negative hepatocytes was apparent. A comparable scattered pattern of expression was seen in tumours stained for β-catenin (Figure 1E). Obviously, the recombination of floxed *Ctnnb1* alleles by Cre was incomplete, leading to a situation where one fraction of tumour cells is *Ctnnb1* KO and therefore GS negative, whereas the other fraction of cells still possesses a non-recombined, mutationally activated *Ctnnb1* allele that drives the expression of the marker protein GS. This heterogeneous phenotype allowed us to investigate two corresponding cell populations within one and the same tumour.

Effect of β-catenin ablation on tumour cell proliferation and apoptosis. For the analysis of tumour cell proliferation, the tumours were first categorised into two groups based on the efficiency of the β-catenin KO, evidenced by loss of the marker protein GS (25–50% or >50% β-catenin-/GS-negative cells, respectively). Tumour by tumour, the fractions of BrdU-incorporating cells were determined separately for both the

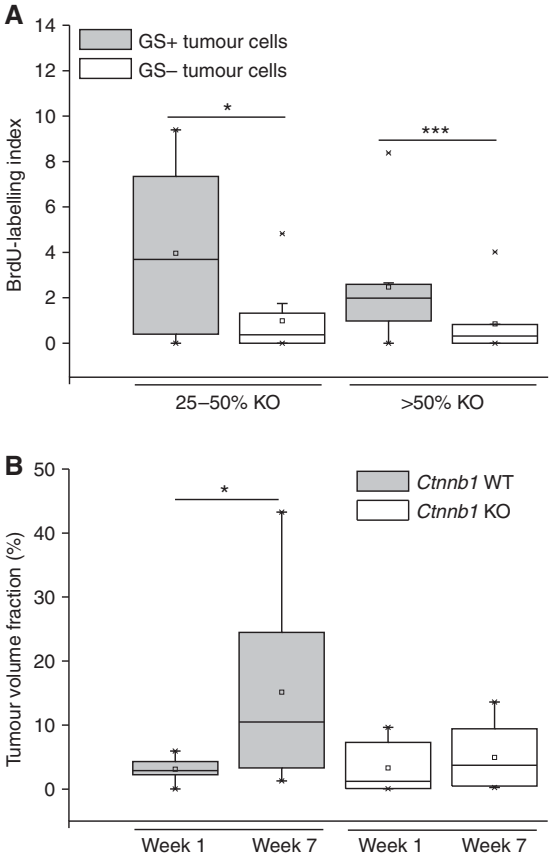

Figure 2. β-catenin deletion diminishes hepatoma cell proliferation. (**A**) BrdU-labelling index of GS-positive and -negative subpopulations in tumours of *Ctnnb1* KO mice after tamoxifen administration. The labelling index is expressed as the percentage of BrdU-positive nuclei in a whole tumour section. On average, 759 nuclei per tumour were counted. Tumours double stained for BrdU and GS are stratified into two groups according to their level of *Ctnnb1* knockout as determined by the percentage of GS-negative tumour cells (25–50% KO, $n=9$; >50% KO, $n=13$). GS-positive tumour subpopulations show a higher proliferation index compared with the GS-negative ones within the same tumour. (**B**) Tumour volume fraction from *Ctnnb1* KO and WT mice 1 and 7 weeks after tamoxifen application. Livers from WT mice show an increase in tumour burden over time, whereas the tumour volume fraction in livers from *Ctnnb1* KO mice is not significantly altered during the 6 weeks' time period. Group sizes: $n=8$ (WT groups; Ctnnb1 KO 7 weeks); and $n=7$ (Ctnnb1 KO 1 week). Statistical differences are indicated by one (*$P<0.05$) or three (***$P<0.001$) asterisks.

GS-positive and the GS-negative hepatoma cell subpopulations. Very small tumours and tumours with a poor recombination rate (that is, <25%) were omitted from the analysis, as a too low number of cells in either of the two subpopulations would lead to inconclusive results. The results of this analysis clearly demonstrated that tumour cells negative for GS exhibit a lower proliferative index as compared with their GS-positive counterparts (Figure 2A). For comparison, GS-positive cells from *Ctnnb1* WT tumours had a mean BrdU-labelling index of 10.3% (data not shown), significantly higher ($P<0.05$; Student's *t*-test) than the values obtained with the GS-positive sub-populations from partially *Ctnnb1*-ablated tumours (4.0% for 25–50% *Ctnnb1* KO; 2.2% for >50% *Ctnnb1* KO). BrdU labelling in surrounding normal tissue was always <1%, irrespective of the genotype. In addition, the tumour volume fraction of *Ctnnb1* WT and KO mice 1 and 7 weeks after tamoxifen treatment was determined

(Figure 2B). After 7 weeks, tumour burden in livers from *Ctnnb1* WT mice was significantly increased, as compared with the tumour burden 6 weeks earlier. In contrast, *Ctnnb1* KO mice showed no obvious alteration in tumour volume fraction over time.

Furthermore, the impact of *Ctnnb1* KO on tumour cell death was investigated 7 weeks after β-catenin ablation. At this time point, the tumour tissue showed intact hepatoma cell nuclei with no obvious signs of necrosis or inflammation. Apoptosis was also almost entirely absent from the tumours in our experiment. TUNEL-stained liver sections revealed no increase in apoptotic cell nuclei after tamoxifen-induced deletion of *Ctnnb1*. Figure 3 shows representative non-apoptotic tumour tissue with residual GS-positive cells from an animal killed 3 weeks after tamoxifen treatment.

Cx32 levels in different tumour subpopulations. Immunohistochemical stainings were performed to analyse the localisation of Cx32, the major gap junction-forming protein in mouse liver, mediating gap junctional intercellular communication (GJIC) (Kumar and Gilula, 1996). Clearly reduced membranous Cx32 was found in GS-positive hepatomas from WT mice (Figure 4A), whereas hepatocytes from normal liver tissue exhibited physiological immunoreactivity for Cx32 in plaque-like structures at the cell membranes (Figure 4B). Interestingly, Cx32 appeared at cell membranes of GS-negative tumour subpopulations after KO of *Ctnnb1* (Figure 4C and D).

DISCUSSION

In the present work, we investigated the impact of a conditional KO of *Ctnnb1* on the growth behaviour of mouse liver tumour cells with an activated Wnt/β-catenin signalling pathway.

In a recent study conducted by Ganzenberg *et al* (2013), tamoxifen-induced, Cre-mediated recombination of the *Ctnnb1* gene led to β-catenin ablation in >99% of hepatocytes in livers from transgenic mice. Using the same mouse strain and treatment protocol, tamoxifen application was less effective in our experiment and the degree of *Ctnnb1* KO varied considerably between different tumours. The reason for this phenomenon is not known. Transthyretin, the gene whose promoter was used to drive the expression of the Cre recombinase in our transgenic system, is not significantly altered in expression in *Ctnnb1*-mutated mouse liver tumours (Stahl *et al*, 2005) and hence altered expression of Cre is most likely not the underlying reason for incomplete recombination. Rather, we suspect that the different age of the mice at the time point of treatment might have had a role. Even more likely, pre-treatment with PB, an inducer of cytochrome P450 3A family enzymes involved in xenobiotic metabolism, might have contributed to the observed effect by inducing tamoxifen metabolism (Kivistö *et al*, 1998).

Nonetheless, although certainly of disadvantage for analyses in tissue homogenates, the coexistence of β-catenin-positive and -negative tumour cells allowed us a direct comparison of the two populations in individual tumours. Our results show that the proliferative index of GS-negative tumour cells was significantly lower than that of the neighbouring GS-positive tumour cells (Figure 2A). This suggests that β-catenin-dependent signalling has a role in regulating cell proliferation in these tumours. Malanchi *et al* (2008) showed that the survival of skin tumours crucially depends on the presence of active β-catenin: the KO of *Ctnnb1* in murine skin tumours with aberrant β-catenin activation resulted in complete tumour regression within 6 weeks. By contrast, however, no indication for the death of hepatoma cells was observed in our analyses (Figure 3). Rather, the loss of β-catenin slowed down the proliferation of the tumour cells. Subsequently, the tumour volume fraction in livers from *Ctnnb1* KO mice did not significantly change within a time period of 6 weeks (Figure 2B). This result points towards differences in the role of β-catenin-dependent signalling for the survival of tumour cells in liver and skin.

An important process involved in the regulation of cell proliferation is Cx32-mediated GJIC. Downregulation of GJIC

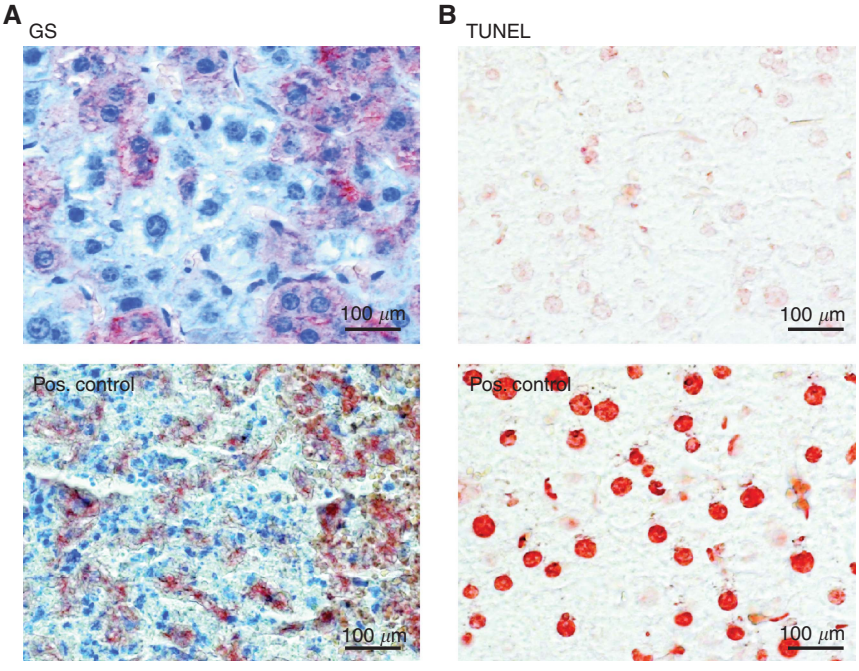

Figure 3. Absence of necrosis and inflammation from *Ctnnb1* KO livers. (**A**) Intact GS-stained tumour tissue taken from a *Ctnnb1* KO animal versus positive 'Pos.' control showing tissue from a highly necrotic tumour infiltrated by immune cells and with remnants of former GS-positive hepatoma cells (sample taken from a previous study by Singh *et al* (2013)). (**B**) Tumour tissue from **A** stained by TUNEL technology reveals the absence of apoptotic cell nuclei after KO of *Ctnnb1*. Positive control was pre-treated with benzonase nuclease to generate free DNA ends. Liver was taken from a *Cre*-positive, *Ctnnb1* KO animal 3 weeks after tamoxifen treatment.

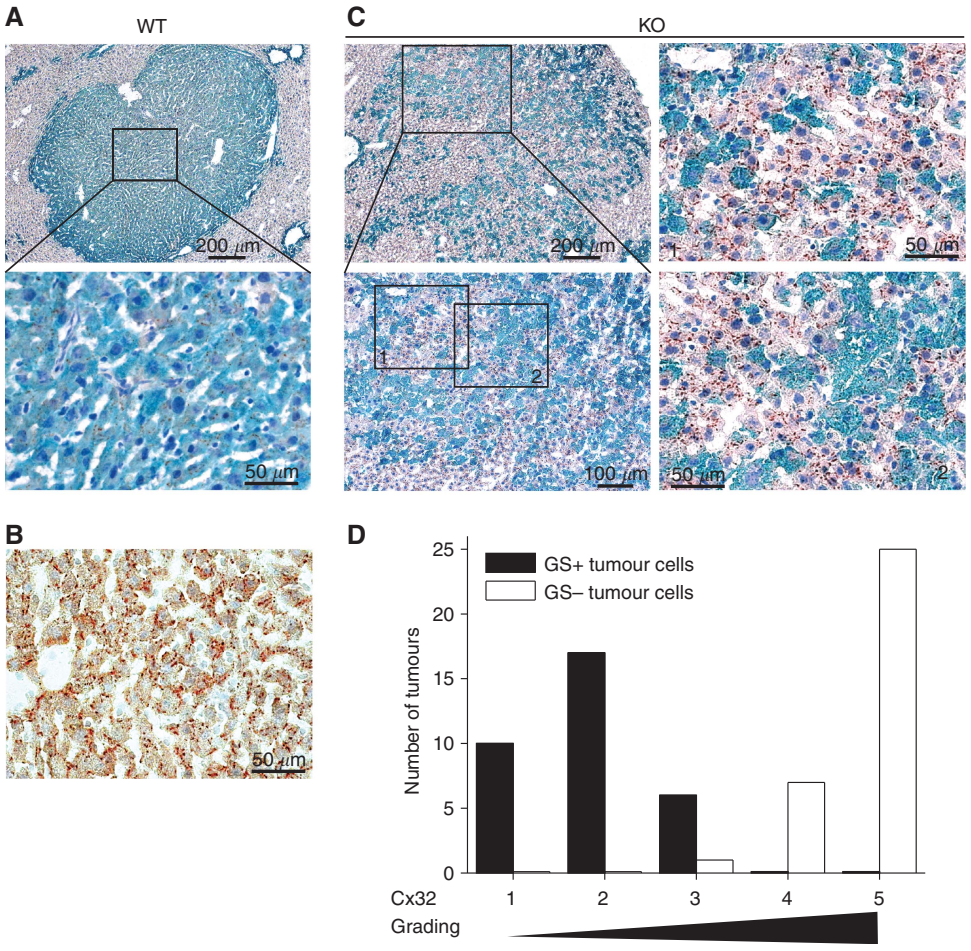

Figure 4. Appearance of Cx32 at membranes of *Ctnnb1* KO tumour cells. (**A**) Reduced Cx32 levels are observable in the membranes of *Ctnnb1*-mutated, GS-positive tumour cells in livers from *Ctnnb1* WT animals. (**B**) In contrast, normal liver tissue shows high membranous Cx32 levels. (**C**) After KO of *Ctnnb1*, Cx32 reappears in the GS-negative tumour cell subpopulations (image details are referred to as 1 and 2). The *Ctnnb1* KO animal was killed 7 weeks after β-catenin ablation. (**D**) Cx32 grading of GS-positive and -negative tumour cells in hepatomas of KO mice revealed higher Cx32 levels in tumour populations lacking GS expression. Tissue slices were double stained for Cx32 and GS and analysed by light microscopy for the presence of Cx32 plaques at the cell membranes of hepatoma cells. For each tumour (in total, $n = 33$ tumours were analysed), the degree of Cx32 expression in the two hepatoma subpopulations was classified from grade 1 (low Cx32 levels) to 5 (high Cx32 levels, comparable to surrounding normal tissue). The numbers of tumours in each class are given in the diagram for the GS-positive and GS-negative sub-populations, respectively.

often occurs during tumorigenesis and triggers proliferation of cancer cells (Chipman *et al*, 2003). Cx32, the major gap junction-forming protein in mouse liver, is absent from the membranes of *Ctnnb1*-mutated tumour cells (Moennikes *et al*, 2000; Marx-Stoelting *et al*, 2008). Our present results confirm that membranous Cx32 protein is decreased in GS-positive tumour cells (Figure 4A). After KO of *Ctnnb1*, Cx32 reappeared at the cell membrane of GS-negative subpopulations (Figure 4C). This indicates that β-catenin-dependent signalling has a role in Cx32-mediated GJIC. Furthermore, it has been reported that the inhibition of GJIC can make tumour cells independent from growth-restraining factors from neighbouring cells (Yamasaki and Naus, 1996; Chipman *et al*, 2003). This raises the possibility that there is a relationship between cell–cell communication and cell proliferation in the examined tumours. Reduced proliferation in the GS-negative hepatoma cell population could be a consequence of recurring Cx32-mediated GJIC. In tumours with more Cx32-positive cells, the remaining β-catenin-positive cells might receive stronger growth-restricting signals from surrounding cells, possibly leading to a stronger inhibition of cell proliferation.

In summary, the present results show that the ablation of β-catenin in mouse liver tumours leads to a significant attenuation of tumour cell proliferation but not to an induction of tumour cell death and hence not to tumour regression. Therefore, β-catenin-dependent signalling seems to be an important factor involved in the regulation of cell proliferation in this experimental system, but its loss is not sufficient to trigger cell death in those tumours.

In addition, GS-positive tumours from WT mice showed massive BrdU incorporation several weeks after the animals were set on a PB-free diet and the tumour volume fraction in respective livers significantly increased over time. This indicates that PB, as a proliferative stimulus, is only required at early stages of tumorigenesis but is no longer needed for maintenance of tumour growth in established *Ctnnb1*-mutated mouse liver tumours.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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